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Molecular Basis of the Glycoprotein-C-Negative Phenotype of Herpes Simplex Virus Type 1 Macroplaque Strain

K. G. DRAPER,[1] R. H. COSTA,[1] G. T.-Y. LEE,[2†] P. G. SPEAR,[2] AND E. K. WAGNER[1]*

Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717[1]; Department of Microbiology, The University of Chicago, Chicago, Illinois 60637[2]

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The basis for the inability of the macroplaque (MP) strain of herpes simplex virus type 1 to express mature glycoprotein C (gC) was examined. RNA transfer (Northern) blot analysis with hybridization probes from the region of the herpes simplex virus type 1 DNA known to encode the gC gene indicated that gC mRNA was produced in MP-infected HeLa cells at levels relative to other mRNAs comparable with that seen in KOS-infected cells. Comparative nucleotide sequence analysis of the gC gene from the MP and KOS strains, coupled with the results of recently reported marker rescue experiments, indicates that the inability of MP to produce gC is due to a frameshift mutation in the gC-coding sequence. Because two different (out-of-phase) open reading frames overlap the gC-coding sequence in the region of the mutation, MP mRNA can encode two gC-related polypeptides. Two polypeptides of the predicted size and precipitable by anti-gC antibodies were produced by in vitro translation of MP mRNA. These polypeptides have not been detected in extracts from infected cells with the same antibodies. Comparative nucleotide sequence analyses led to several corrections in the published sequence for the gC gene and the 17,800-molecular-weight polypeptide gene just to the right in KOS DNA. These relatively minor effects on the predicted amino code sequence of gC are tabulated.

The region of the herpes simplex virus type 1 (HSV-1) genome encoding the glycoprotein C (gC) has been well characterized (11, 12, 16). Analysis of the transcription of the mRNAs encoding gC and a neighboring 17,800-molecular-weight protein of unknown function demonstrates a number of the features of HSV transcription that have been the subject of two recent reviews (27; E. Wagner, *in* B. Roizman, ed., *The Herpesviruses*, vol. 3, in press). Among these features are the occurrence of sequences recognizable as putative promoters just upstream of the mRNA cap sites and a lack of splicing in the most abundant mRNAs. Of further interest is the occurrence of a number of mRNAs of much lower abundance that appear to differ from mature gC mRNA only in having been spliced (11, 12). Thus, continued analysis of transcription of the gC gene will provide information of direct bearing on the mechanism of HSV gene expression.

Although most clinical isolates of HSV express gC, and gC-1 has recently been shown to bind the third component of complement (10a), its precise biological function is unknown. The macroplaque (MP) strain of HSV-1 lacks the ability to express mature gC as described previously (13, 17). Other strains of HSV-1 (4, 8, 24) and HSV-2 (29) have been characterized that also fail to express this protein. Therefore, the expression of the complete gC protein is not necessary for productive infection of HSV-1 or HSV-2 in cell culture.

Recent studies suggest that the mutation responsible for the gC^- phenotype of MP is in the structural gene for gC (K. L. Pogue-Geile, G. T.-Y. Lee, S. K. Shapiro, and P. G. Spear, Virology, in press). Specifically, marker rescue experiments showed that MP could be converted to the gC^+ phenotype by a cloned *Sal*I fragment containing the HSV-1 (F) gC gene (wild-type). Conversely, a cloned fragment of MP DNA containing the gC locus converted wild-type HSV-1 to the gC^- phenotype.

Comparative restriction endonuclease fragment patterns of different HSV-1 and HSV-2 strains showed reproducible sequence variation (reviewed in reference 26). The frequency of nucleotide sequence differences in specific genes has been calculated to be on the order of 1.5 percent between individual clinical isolates of HSV-1, based on comparative interstrain base sequence analysis of the thymidine kinase (*tk*) gene (29) and a portion of the 38,000-molecular-weight ribonucleotide reductase protein (10, 22). In these cases, the viruses examined expressed an identified polypeptide product. The lack of expression of the gC protein in the MP strain provides a different system that could be under even less constraints toward sequence divergence.

In the present study, we have used mRNA mapping, in vitro translation, and nucleotide sequence analysis to examine the lesion in the gC gene of the MP strain of HSV-1. The relative amount of gC mRNA expressed in the MP strain appears to be comparable to that produced in the KOS strain, which expresses gC protein. In the MP strain, there is an additional base inserted 290 bases downstream of the translation initiator. Because of out-of-phase reading frames that overlap the gC-coding sequence, two different gC-related polypeptides could be produced by MP as a result of the frameshift mutation. One of these is predicted to have the same amino acid sequence as gC upstream of the mutation and an additional 21 amino acids downstream. Conversely, the other is predicted to have the same amino acid sequence as gC downstream of the mutation and an unrelated sequence of 27 amino acids upstream. Polypeptide products of size expected for both of these truncated polypeptides were detected by in vitro translation.

The frequency of sequence divergence between the KOS and MP strains in the C-terminal region of the gC translational frame is somewhat less than in the N-terminal region. The overall frequency of change is on the order of 1.5%. Thus, the lack of expression of normal gC and extensive passage of

* Corresponding author.
† Present address: School of Medicine, University of Miami, Miami, FL 33101.

the MP strain in cell cultures does not appear to have resulted in a notable increase in the rate of base sequence divergence either in the promoter for the gC mRNA or in the structural gene for this protein.

MATERIALS AND METHODS

Cells and virus. Monolayer cultures of HeLa cells were grown at 37°C in Eagle minimal essential medium containing 10% calf serum, penicillin, and streptomycin. Monolayer cultures of HEp-2 cells were grown on 37°C in Dulbecco modified Eagle minimal essential medium containing 10% fetal calf serum, penicillin, and streptomycin. Plaque-purified virus of either the KOS strain or the MP strain of HSV-1 was used for the designated infections.

Enzymes. All restriction enzymes and bacterial alkaline phosphatase were obtained from Bethesda Research Laboratories. Digestions were carried out in buffers recommended by the supplier. Phage T4 polynucleotide kinase (Bethesda Research Laboratories) was used for 5′ end labeling as described by Maxam and Gilbert (20).

Isolation, labeling, and size fractionation of polyribosomal RNA. Monolayer cultures of HeLa cells (2×10^7 cells per flask) were infected for 30 min at a multiplicity of 10 PFU of virus per cell in phosphate-buffered saline containing 0.1% glucose and 1.0% fetal calf serum. Polyribosomes were isolated from the cytoplasm of HSV-1-infected cells by the magnesium precipitation method of Palmiter (23). Polyadenylic acid-containing [poly(A)] mRNA was isolated from total rRNA by oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc.) chromatography. This is referred to as HSV poly(A) mRNA. Details of this procedure were presented elsewhere (6, 12). RNA was isolated at 6 h postinfection. RNA was size fractionated by electrophoresis on 1.4% agarose gels containing 10 mM methylmercury hydroxide (3) as previously described (1, 2, 6, 7, 11, 12).

Recombinant DNA. All recombinant DNA clones described in this paper were derived from either *Bgl*II fragment N (0.233 to 0.271 map units [m.u.]), *Hin*dIII fragment L (0.592 to 0.647 m.u.), and *Bam*HI fragment I (0.602 to 0.643 m.u.) of the KOS strain of HSV-1 or *Bam*HI fragment I (0.602 to 0.643 m.u.) of the MP strain of HSV-1, cloned in pBR322. Five subclones were used: *Bam*HI fragment A′ (0.258 to 0.268 m.u.), *Sal*I-*Eco*RI fragment T-A (0.621 to 0.633 m.u.), and *Eco*RI-*Bam*HI fragment I-I (0.633 to 0.643 m.u.) of the KOS strain or *Sal*I-*Eco*RI fragment T-A (0.621 to 0.633 m.u.) and *Eco*RI-*Bam*HI fragment I-I (0.633 to 0.643 m.u.) of the MP strain. Procedures for cloning HSV-1 DNA fragments in the pBR322 vector were described previously (1, 7). DNA fragments cloned were named as described previously and located by their map coordinates on the P arrangement of the HSV-1 genome (2).

In situ Northern RNA blots. Unless noted otherwise, 5-μg samples of HSV poly(A) mRNA were fractionated on methylmercury gels and dried onto Whatman 3mm paper with vacuum as previously described (12). The agarose film was floated off the paper in water and hybridized with appropriate nick-translated ^{32}P-labeled DNA probes in 50% formamide containing 0.4 M Na^+, 0.1 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 8.0), 0.005 M EDTA, and Denhardt solution (9) at 50°C for 36 h. Blots were rinsed at 50°C. The first two rinses were in 50% formamide–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS). The last rinse was in 0.1× SSC–0.1% SDS. Autoradiography was on Kodak XRP film with or without intensifying screens as needed.

In vitro ^{32}P-labeled DNA was made by nick translating appropriate DNA clones with DNA polymerase I, DNase I (Boehringer-Mannheim Corp.), and 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp.).

Isolation of restriction fragment-specific mRNA. Restriction fragment-specific mRNA was isolated from HSV poly(A) mRNA by preparative hybridization to the appropriate DNA covalently coupled to cellulose. Details of coupling of DNA to cellulose and preparative hybridization were as described previously (1, 2).

Nucleotide sequencing. As described previously (10, 12), nucleotide sequence analysis was carried out by the procedure of Maxam and Gilbert (20).

In vitro translation. Translation of size-fractionated viral mRNA was carried out in vitro by using a micrococcal nuclease-treated rabbit reticulocyte system (New England Nuclear Corp.) with [^{35}S]methionine (>800 Ci/mmol) as the radioactive amino acid. Details of the procedure and fractionation of polypeptides in SDS-acrylamide gels by the method of Laemmli (14) were described in several previous papers. In one set of experiments, proteins were fractionated by modification of the method of Shapiro et al. (25). Gels were treated with En^{3}Hance (New England Nuclear Corp.) and dried with vacuum at 60°C, and radioactive bands were localized by autoradiography with Kodak XRP film. Exposure was for 3 to 5 days at −70°C.

Immune precipitation of in vitro translation products was performed as described by Matthews et al. (19). One-half of the RNase-treated translation product (14 μl) was diluted with an equal volume of 2× lysis buffer and incubated with 4 μl of env-1 serum, a polyvalent antibody to HSV-1 envelope protein (5, 12). Lysis buffer is 20 mM Tris-hydrochloride (pH 7.4), 50 mM NaCl, 10 mM methionine, 0.5% Nonidet P-40, 0.5% sodium desoxycholate, and 0.1% SDS. After 1 h on ice, 75 μl of a 10% suspension of pro-A Sepharose beads (Pharmacia Fine Chemicals) in 50 mM Tris-hydrochloride (pH 7.5)–150 mM NaCl–5 mM disodium EDTA–0.2% sodium azide was added, and the suspension was incubated for a further 30 min on ice with frequent mixing. The pro-A Sepharose beads with the immune complex adsorbed were then deposited by 1 min of centrifugation in an Eppendorf microcentrifuge, and the pellet was suspended in 200 μl of lysis buffer and centrifuged for 5 min through a 1-ml pad of lysis buffer containing 1 M sucrose. The Sepharose was then washed by suspension in 0.5 ml of 10 mM Tris-hydrochloride (pH 7.4)–150 mM NaCl–10 mM methionine–0.2% Nonidet P-40–0.1% SDS followed by recentrifugation. After five washes, the Sepharose pellet was incubated with 20 μl of a buffer containing 0.1 M Tris-hydrochloride (pH 7.0), 3% SDS, 10% β-mercaptoethanol, and 20% (vol/vol) glycerol. The suspension was heated to 95°C for 2 min, and the Sepharose was pelleted by centrifugation. The supernatant containing released immunoglobulin and any translation product that it had adsorbed was then loaded onto SDS-acrylamide gels for size fractionation.

For the experiment shown in Fig. 7 below, HEP-2 cells were infected with HSV-1 (mP), HSV-1 (MP), or HSV-1 (F) at 20 PFU per cell and incubated at 34°C until about 14 h after infection. Then, cytoplasmic poly(A) RNA was isolated and translated in vitro as described by Lee et al. (15, 16). A monospecific polyclonal anti-gC serum (R-24) was used to precipitate gC-related polypeptides from the products of in vitro translation. Preparation of this antiserum against SDS-denatured gC and demonstration that it selectively precipitated gC polypeptide made in vitro were described previously (16).

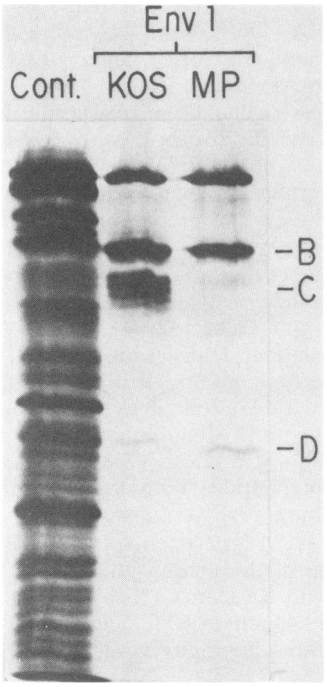

FIG. 1. Immunoprecipitation of infected cell proteins with antiserum env-1. Autoradiography is of a denaturing SDS-acrylamide gel (14). Nomenclature of HSV-1 glycoproteins is as previously described (26). Cells were labeled for 15 min with [^{35}S]methionine at 7 h postinfection. Tracks are as follows: Cont., cytoplasmic [^{35}S]methionine-labeled infected cell protein; KOS, immunoprecipitation of cytoplasmic, ^{35}S-labeled HSV-1 (KOS strain)-infected cell protein with polyclonal antiserum env-1; MP, immunoprecipitation of cytoplasmic, ^{35}S-labeled, HSV-1 (MP strain)-infected cell protein with polyclonal antiserum env-1.

Preparation of radioactive protein markers. [^{35}S]methionine-labeled, cytoplasmic infected-cell protein was isolated as described previously (6). Cultures (6×10^6 cells) of HeLa cells were incubated for 15 min at 7 h postinfection with 200 μCi of [^{35}S]methionine (400 Ci/mmol; New England Nuclear Corp.) in 4 ml of methionine-free Eagle minimal essential medium.

RESULTS

MP strain produces normal amounts of gC-specific mRNA. The gC$^-$ phenotype of the MP strain of HSV-1 is readily seen by using a short pulse of radioactive methionine and a polyspecific antiserum reactive with the viral envelope glycoproteins. In the experiment shown in Fig. 1, cells were infected with either the KOS strain or the MP strain of HSV-1. They were then incubated with [^{35}S]methionine for 15 min at 6 h postinfection. The total infected cell protein was isolated and incubated with the env-1 polyclonal antiserum described by Cohen et al. (5) and Frink et al. (12). Immune reactive protein was recovered by batch chromatography with pro-A Sepharose and fractionated by denaturing acrylamide gel electrophoresis. Major glycoproteins corresponding to gB and gD are clearly seen to be produced after infection by both strains of HSV-1. However, the major series of bands migrating as immature gC is missing from the MP-infected cells.

It should be noted that there is a small amount of radioactivity migrating with the rate expected for gC in MP-infected cells. The experiments described here demonstrate that this is probably not due to small levels of gC synthesis taking place in the MP-infected cells. This material was not investigated further in the present series of experiments.

The transcription map of HSV-1 in the region of the genome encoding gC was described recently (11, 12), and a summary of that transcription map is shown in Fig. 2. The major gC reading frame and alternate reading frames, including a truncated one, utilizable by a spliced mRNA are indicated.

The defect in the MP strain's ability to synthesize gC could be at the level of expression of gC mRNA or at the level of the translation of that mRNA into a recognizable protein product. We used RNA transfer (Northern) blots of RNA isolated from polyribosomes of cells infected with the MP strain to determine that mRNA corresponding to that encoding normal gC is made in normal amounts. We used the DNA fragment between the *Eco*RI site at 0.633 m.u. and the *Bam*HI site at 0.643 m.u. as a probe to detect gC-specific mRNA. mRNA species migrating with a rate corresponding to a polyadenylated size of 2.7 and 1 kilobases were seen in MP-infected cells (Fig. 3A). No difference in the rate of migration of these mRNA species could be seen in comparison with the same species from KOS-infected cells (compare tracks i and ii of Fig. 3A). An RNA transfer blot of a larger amount of mRNA isolated from MP-infected cells also indicated that the minor transcripts seen in this region due to low levels of splicing are present in the same relative amounts (Fig. 3A, track iii).

The two lanes of RNA shown in Fig. 3A (tracks i and ii) were loaded with 5 μg of infected cell poly(A) polyribosomal RNA. The data were thus consistent with a reduced amount of gC mRNA being produced in the MP-infected cells. We found, however, that MP produces a reduced amount of other mRNAs compared with KOS. This conclusion is based on the following experiment measuring the relative amount of another mRNA species in the mRNA isolated from KOS- and MP-infected cells. We determined the amount of the 6-kilobase mRNA encoding VP-5 (6, 7) in the two mRNA populations by using *Bam*HI fragment A′ (0.258 to 0.268 m.u.) as a hybridization probe on an RNA transfer blot of 5 μg of KOS and MP mRNA fractionated in parallel. There is less 6-kilobase mRNA recoverable in the poly(A)-containing

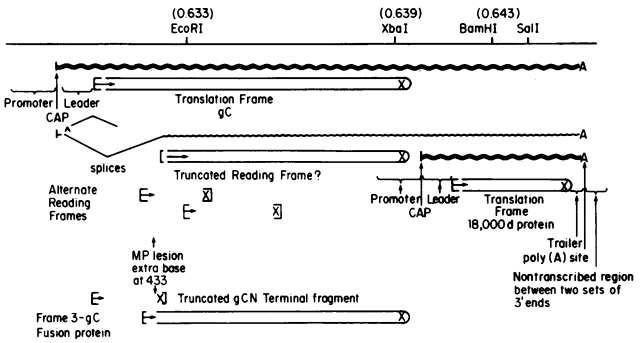

FIG. 2. Transcription map of HSV-1 in the region encoding gC. The diagram illustrates the locations of relevant restriction endonuclease sites (*Eco*RI, *Xba*I, and *Bam*HI) and their m.u. positions (0.633, 0.639, 0.643, respectively) on the prototypic (P) arrangement of the HSV-1 (KOS) genome. The cap sites of the two major mRNA species (indicated by bold lines), as well as representative exon boundaries of minor mRNA species are as described previously (12). Approximate translation reading frames are illustrated. Alterations in frames due to the extra base inserted in the MP strain (see below) are indicated also. Start (E-) and stop (X) signals are derived from sequence analysis.

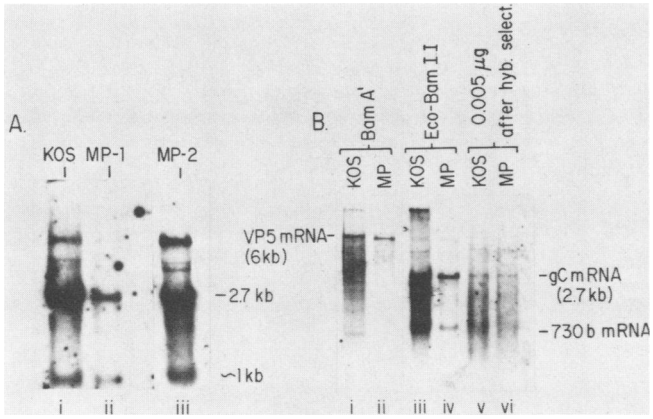

FIG. 3. In situ RNA (Northern) blots comparing levels of mRNA encoded by HSV-1 (KOS strain) and HSV-1 (MP strain). Samples of HSV late poly(A) mRNA from infected cells were fractionated on methylmercury-containing agarose gels and immobilized by drying in vacuo. The RNA was detected by hybridization with specific nick-translated DNA probes as indicated. Sizes shown were determined by the position of HeLa cell rRNA markers (not shown) as described previously (1). Unless otherwise noted, 5 μg of polysomal poly(A) mRNA was fractionated in each lane. (A) Tracks: i, KOS strain mRNA species hybridizing to a probe made from *Eco*RI-*Bam*HI fragment I-I (0.633 to 0.643 map m.u.); ii, MP strain mRNA species hybridizing to the same probe; iii, same as track ii, except 15 μg of poly(A) RNA was loaded onto gel. (B) Tracks: i, KOS strain mRNA species hybridizing to a probe made from *Bam*HI fragment A' (0.258 to 0.268 m.u.); ii, MP strain mRNA species hybridizing to the same probe; iii, KOS strain mRNA species hybridizing to a probe made from *Eco*RI-*Bam*HI fragment I-I (0.633 to 0.643 m.u.); iv, MP strain mRNA species hybridizing to this probe; v, 0.005 μg of hybrid purified KOS gC region-specific mRNA species hybridizing to a probe made from *Eco*RI-*Bam*HI fragment I-I; vi, 0.005 μg of hybrid purified MP strain gC region-specific mRNA species hybridizing to this probe.

mRNA preparations from MP-infected cells than there is from KOS-infected cells (Fig. 3B). A parallel fractionation of gC mRNA probed with *Eco*RI-*Bam*HI fragment II (0.633 to 0.643 m.u.) demonstrates the same relative abundance of the two specific mRNAs in the KOS and MP RNA preparations. We conclude that our consistent finding of relatively less HSV-specific mRNA in the MP-infected cell RNA isolates is not a specific problem in the production of any given mRNA, but is more likely due to the fact that the MP strain of HSV-1 was not extensively adapted to growth in HeLa cells before the experiments described here. Further, alterations in membranes caused by the fusion-inducing strain MP may result in lowered RNA and protein synthesis.

gC gene of the MP strain of HSV-1 has altered translational reading frames due to the addition of an extra base. The data discussed above suggested that the lesion in the MP strain of HSV-1 resulting in lack of synthesis of gC was at the level of translation of MP gC mRNA. Comparative sequence analysis of the DNA of both strains of the virus and in vitro translation (see below) demonstrated this to be the case. To compare the base sequence of the DNA between the two strains, we did parallel sequence ladders of the DNA between the 120 bases 5′ of the cap site for gC mRNA down to the *Bam*HI site at 0.643, which lies 300 bases 3′ of the cap site of the mRNA encoding the 17,800-molecular-weight protein. In so doing, we noted several errors in the sequence of this region published previously (12). None of the corrections significantly alters the basic results of the earlier publication. However, there are some changes in the predicted C-terminal amino acid sequence of the gC protein and the predicted N-terminal amino acid sequence of the 17,800-molecular-weight protein. The corrections to the published sequence are shown in Table 1. Here, as in the discussion below, all sequence numbers refer to the sequence of Fig. 2 of Frink et al. (12).

The parallel sequence studies demonstrated 32 positions where sequence differences occur between the MP and KOS strains of HSV-1 in the 2,210 bases examined. These differences and their predicted results on the proteins encoded in this portion of the HSV-1 genome are shown in Table 2. The frequency of base differences is approximately 1.4%, which is a value similar to the intratypic variation seen in the HSV-1 *tk* gene (21, 28) and in the region of the HSV-1 genome encoding the early (β) 140,000-molecular-weight and 38,000-molecular-weight proteins (10, 22).

The difference that results in the MP strain's lack of expression of gC is the addition of an extra G:C pair to the string of 7 G:C pairs between base numbers 433 and 439 (Fig. 4). This alters the translational reading frame as shown in the lower portion of Fig. 2. Two major polypeptide products are potentially encoded in the MP gC mRNA, one containing the N-terminal 20% of the gC protein fused to 21 amino acids encoded in another reading frame, and the other containing an altered N terminus due to initiation at a frame 3 ATG and the normal C-terminal 80% of the gC protein.

gC mRNA isolated from MP-infected cells encodes novel gC-related polypeptides observable by in vitro translation. We used *Eco*RI-*Bam*HI fragment I-I DNA (0.633 to 0.643 m.u.) bound to cellulose as a reagent to isolate mRNA encoded in the region of the gC gene (gC region mRNA) for in vitro translation studies. We hybridized approximately three times as much infected cell mRNA from MP-infected cells as from a corresponding KOS infection with the standard amount of DNA bound to cellulose to insure that we recovered sufficient gC mRNA for efficient in vitro translation. A sample (ca. 0.005 μg) of the gC region mRNA (2.7-kilobase gC mRNA and the neighboring 1-kilobase mRNA) from MP-infected cells was fractionated in parallel with the same amount of gC region mRNA from KOS-infected cells. The essential equivalence in amounts is shown in Fig. 3B (tracks v and vi).

TABLE 1. Corrections in the published sequence for the HSV-1 (KOS) DNA encoding gC

Base nos.[a]	Correction	Alteration in predicted amino acid sequence[b]
1,471–1,472	CT to TC	gC amino acid 443 (panel A) try to arg
1,668–1,669	GC to GTC	gC terminates at TAA at 1,678; thus, predicted C-terminal amino acid sequence from 509 is His-Arg-Arg-COOH
1,729–1,732	CTTC to CTC	No change
1,807	G to C	No change
1,888–1,890	AGC to ACGC	17,800-molecular-weight protein encoded by 730-base mRNA (panel B) brings phase 3 initiator at 1,866 in phase with phase 2-ATG at 1,922 and yields 19 extra N-terminal amino acids: H_2N-Met-Pro-Leu-Arg-Ala-Ser-Glu-His-Ala-Tyr-Arg-Pro-Leu-Gly-Pro-Gly-Thr-Pro-Pro

[a] Data from Fig. 2 of reference 12.
[b] Data from Fig. 5 of reference 12.

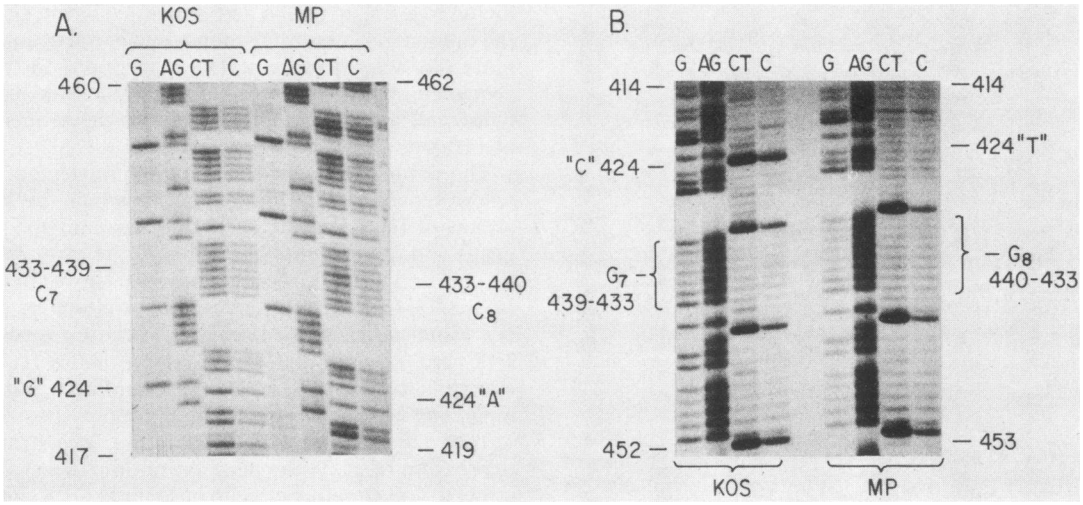

FIG. 4. Precise localization of the frameshift lesion in the gC gene of HSV-1 MP strain. DNA sequencing was by the method of Maxam and Gilbert (20). Base numbers are taken from Frink et al. (12). (A) DNA sequence ladders comparing the gC region base sequences of the *Sal*I-*Eco*RI fragments T-A (0.621 to 0.633 m.u.) of the KOS and MP strains of HSV-1. The sequence presented is that of the coding strand in the region around nucleotides 424 through 440. (B) DNA sequence ladders comparing the gC region base sequences of the *Sal*I-*Eco*RI fragments T-A of the KOS and MP strains of HSV-1. The sequence presented is that of the noncoding strand in the region around nucleotides 424 through 440.

As shown previously (12), translation of gC region mRNA from KOS-infected cells yields the gC polypeptide migrating with a rate corresponding to a nominal size of 69,000 molecular weight, the 17,800-molecular-weight translation product of the neighboring 1-kilobase mRNA, and lesser amounts of intermediate-sized polypeptides that may be translation products of the minor spliced mRNA species (Fig. 5, track i). Here, the 17,800-molecular-weight polypeptide is not readily visible, but is clear in the original autoradiography. Translation of an equivalent amount of the region-specific mRNA from MP-infected cells is shown in Fig. 5 (track v). The 17,800-molecular-weight protein is evident, along with a minor amount of an intermediate-sized polypeptide (ca. 60,000 molecular weight), but no 69,000-molecular-weight protein is seen.

The translation products of the MP RNA were also fractionated on a 15% acrylamide–6 M urea gel to resolve low-molecular-weight polypeptides (Fig. 6). The 17,800-molecular-weight polypeptide is clearly seen after a 2-day exposure of the total fractionated translation product (Fig. 6A, track ii). Several other protein bands migrating more rapidly than this protein were seen with difficulty. Long (3-week) exposures of the fractionated translation products of MP gC region mRNA reactive with anti-env-1 serum on 15% acrylamide–6 M urea gels shows the recovery of the polypeptide migrating more rapidly than the 17,800-molecular-weight protein (Fig. 6B, track iii). We suggest that this could be the truncated polypeptide containing sequences related to the N terminus of gC and predicted to be encoded by the MP gC mRNA. Such a protein has a nominal molecular weight of 12,000. However, the fact that it contains 21 proline residues out of 120 amino acids (18%) suggests that it would migrate anomalously in acrylamide gels (12).

No other translation products reactive with the env-1 antiserum were seen. However, the use of a monospecific, polyclonal antiserum against gC (R-24; 15, 16) permitted detection of a larger (50,000- to 60,000-molecular weight) translation product of MP gC mRNA. In this experiment,

TABLE 2. Differences between KOS and MP in the nucleotide sequence of the gC gene

Base nos.[a]	Nucleotide		Predicted amino acid differences between KOS and MP
	KOS	MP	
−116	G	A	
−114	T	C	
−6	A	C	
118	G	T	
128	A	G	
154	T	G	gC Pro to Pro (no change)
185	G	A	gC Gly to Ser
290	T	A	gC Ser to Thr
392	A	G	gC Ser to Gly
424	G	A	gC Thr to Thr (no change)
433–439	C_7	C_8	gC changes frame to terminate at base 503 and results in fusion of frame 3 (initiated at base 354) with C-terminal part of gC
492	G	A	gC Gly to Asp
510	C	T	gC Pro to Leu
538–540	C_3	T_3	gC Pro to Leu
633	T	C	gC Met to Thr
689	A	G	gC Ilu to Val
1,126	A	C	gC Thr to Thr (no change)
1,201	T	C	gC His to His (no change)
1,270	C	T	gC Ilu to Ilu (no change)
1,291	G	T	gC Arg to Arg (no change)
1,495	G	C	gC Gly to Gly (no change)
1,528	G	A	gC GlN to GlN (no change)
1,685	G	A	None
1,689–1,694	C_6	C_{10}	None
1,725–1,730	C_6	C_5	None
1,775	T	C	None
1,855	A	G	None
1,857	A	G	None
1,860	C	G	None
1,906	C	T	Gly to Gly (no change)
1,922	A	G	Met to Val
1,990	T	C	Ilu to Ilu (no change)

[a] Data from Fig. 2 of reference 12.

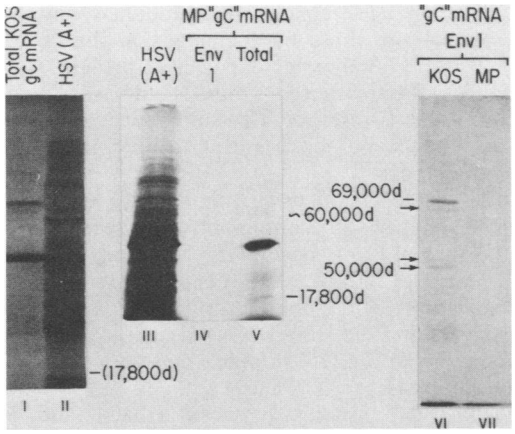

FIG. 5. In vitro translation of mRNAs specific to *Eco*RI-*Bam*HI fragment I-I DNA (0.633 to 0.643 m.u.) followed by antibody precipitation with env-1. Hybrid-selected mRNA was in vitro translated in a micrococcal nuclease-treated rabbit reticulocyte system with [^{35}S]methionine used as a label, and the protein products were fractionated on an SDS–9% acrylamide gel. Sizes of proteins were determined by comigration with adenovirus mRNA translation products (not shown) as described previously (2, 6). Tracks: i, in vitro translation products of *Eco*RI-*Bam*HI fragment I-I DNA-specific mRNA (KOS strain); ii and iii, in vitro translation products of HSV-1-infected cell mRNA; iv, in vitro translation products of *Eco*RI-*Bam*HI fragment I-I DNA-specific mRNA (MP strain) precipitated with env-1 antiserum; v, total in vitro translation products of *Eco*RI-*Bam*HI fragment I-I DNA-specific mRNA (MP strain); vi and vii, in vitro translation products of *Eco*RI-*Bam*HI fragment I-I DNA-specific mRNA (KOS strain and MP strain, respectively) precipitated with env-1 antiserum.

total polyribosomal poly(A) mRNA from cells infected with the mP, MP, and F strains of HSV-1 was translated in vitro and reacted with the R-24 serum. The translation product of the mP and F strains (wild type) yielded an immunoreactive band migrating with a nominal size of 69,000 to 72,000 molecular weight, the size of in vitro-translated gC (Fig. 7). The translation product of MP mRNA is significantly smaller, migrating with a nominal size of 50,000 to 60,000 molecular weight. This is a reasonable candidate for the predicted frame 3 N-terminal–gC–C-terminal fusion protein shown in Fig. 2.

DISCUSSION

The data presented here unambiguously define the genetic lesion affecting gC expression in the MP strain of HSV-1. Because the laboratory history of this strain is known and a sister strain (mP) exists that contains a functional gC gene, a number of interesting coevolutionary studies are feasible. Further, the portion of the HSV-1 genome encoding the leader sequences, translational reading frame, and 3′ untranslated sequences provide other regions suitable for genetic manipulations and constructions similar to those carried out in the region near the *tk* gene.

The polyclonal, polyspecific env-1 serum used in this study to examine gC synthesis in MP-infected cells does react with some infected cell protein that migrates with a rate similar to gC after a 15-min pulse label (Fig. 1). Although this material could be a precursor to one of the larger, mature glycoproteins, it is also possible that it is another, as yet uncharacterized component of the membranes of the HSV-1-infected cell. It is clear that there is at least one additional glycoprotein detectable in HSV-2-infected cells compared with HSV-1-infected cells (18, 24). Further, L. Hall and E. Wagner (unpublished data) found that the in vitro translation product of an mRNA mapped to the right of gC mRNA reacts with env-1 serum. Obviously, the MP strain of the virus would be a useful one in attempting to characterize additional HSV-1 membrane-associated proteins.

The steady-state level of gC mRNA is lower in MP-infected cells than in KOS-infected cells, but the relative amount of gC mRNA vis a vis other HSV-1 mRNA species is not different. This conclusion is based upon the measurement of the ratio of gC mRNA to VP-5 mRNA in both infections (Fig. 3B). Thus, the MP mRNA transcribed from the region of the gC gene is evidently as stable as is the normal gC mRNA. Questions about the expression and stability of the predicted gC-related polypeptides in MP-infected cells have not been resolved. They have not been observed in infected cells. One of the abnormal proteins would be excreted into the culture medium, and the proper monospecific or monoclonal antibody directed against gC could potentially detect it. It should be noted, however, that T. C. Holland, F. Homa, S. Marlin, M. Levine, and J. Glorioso (unpublished data) have examined a number of variants of HSV-1 with in vitro-mutagenized gC genes and reported that there appears to be a minimum size of truncated polypeptide necessary for stability and secretion.

Because the frameshift mutation in MP occurs in a region where three open reading frames overlap (12), the possibility exists that two other gene products in addition to gC are altered by the mutation. Other polypeptides that could be translated from spliced mRNAs overlapping the major gC transcript should not be affected by the mutation. These minor mRNAs appear to be expressed in the same relative proportion to the gC mRNA during MP infection as seen during KOS infection (Fig. 3A). It was suggested that a minor in vitro translation product seen migrating with a rate corresponding to 50,000 molecular weight could be encoded by one of the spliced mRNAs and could share the C-terminal portion of the gC translation reading frame since this polypeptide reacted with the env-1 serum (12). This band and

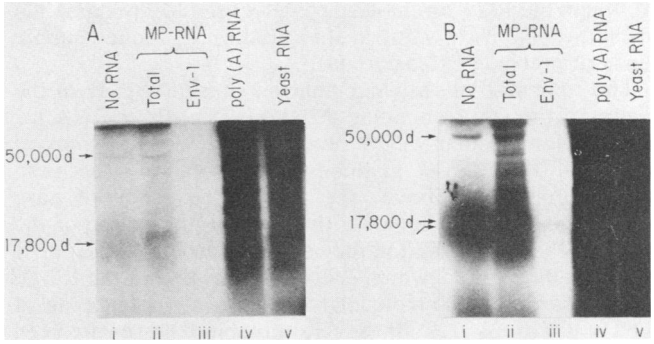

FIG. 6. Immunoprecipitation of truncated proteins related to gC after in vitro translation of *Eco*RI-*Bam*HI fragment I-I DNA-specific MP strain mRNA. Autoradiographs are on 15% acrylamide—6 M urea gels (25). The size of the 17,800-molecular-weight protein was determined by comigration with adenovirus mRNA translation products as described previously (12). (A) Tracks: i, no RNA control; ii, total in vitro translation products of *Eco*RI-*Bam*HI fragment I-I DNA-specific MP strain mRNA; iii, in vitro translation products of *Eco*RI-*Bam*HI fragment I-I DNA-specific MP strain mRNA precipitated with env-1 antiserum; iv, in vitro translation products of HSV-1-infected cell mRNA; v, in vitro translation products of *Saccharomyces cerevisiae* mRNA. (B) All tracks the same as in panel A, except autoradiography was for 2 weeks.

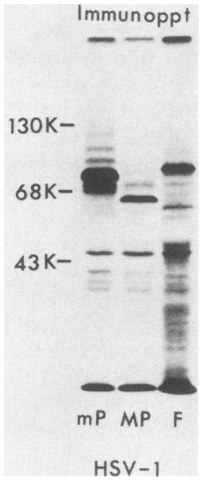

FIG. 7. Detection of translation frame 3 C-terminal gC fusion protein encoded by MP gC mRNA. Total poly(A) polyribosomal mRNA was translated in vitro and reacted with R-24 serum as described in the text and by Lee et al. (16). The sizes shown in this figure are based on the size standards described by Lee et al. (15, 16). They are somewhat larger than would be determined with standards described elsewhere (this article and in reference 12).

minor amounts of several other radioactive polypeptides reactive with anti-env-1 serum are expressed upon translation of the gC mRNA from KOS-infected cells, but they are not present in readily detectable amounts in the immune-precipitated products of translation of the corresponding MP mRNA species (Fig. 5, tracks vi and vii). These results suggest that the minor immune-reactive polypeptides seen in translation of KOS gC mRNA could be artifacts. It may be possible to detect the translation products of the minor mRNAs by using material from MP-infected cells and monoclonal antibodies directed against epitopes in the C-terminal portion of the gC protein when such become available. Although the R-24 serum used to detect the MP polypeptide in Fig. 7 could be of value in such studies, its limited supply tends to preclude extensive use.

The comparative sequence analysis presented here of the region of the DNA encoding gC and part of the mRNA for the 17,800-molecular-weight protein is the second extensive region of the HSV-1 genome to be compared between strains. As noted above, the overall frequency of base differences is quite similar to that seen in the region of the genome encoding *tk* and in the 667 bases around the *Hin*dIII region at 0.592 m.u., which encodes the 5′ end of the 1.2-kb mRNA for a 38,000-molecular-weight protein mapping at 0.60 m.u. (10, 21, 22, 28). Several notable features are seen in the comparative data of Table 2. Excluding the inserted base, there are 16 differences in the 1,536-base gC translational reading frame. Eight of the changes are in the 434 bases between the translation initiation codon and the *Eco*RI site at nucleotide 588. Only two of the eight base changes in the C-terminal regions 3′ from the *Eco*RI site result in a change in predicted amino acid, whereas six of the eight changes alter the predicted amino acid in the N-terminal portion of the gC protein. In the case of the HSV-1 *tk* gene, seven of 19 single-base changes result in changes in predicted amino acid sequence (31). In the reading frame of the 38,000-molecular-weight protein mapping at 0.60 m.u., three of four base changes change the predicted amino acid. Finally, one of the three base changes in the N-terminal portion of the 17,800-molecular-weight protein surveyed here changes the predicted amino acid. All these data suggest that the C-terminal two-thirds of the HSV-1 gC protein is under some constraint as to its ability to accept amino acid changes.

All three of the instances in this study where there are additions or deletions in the number of nucleotides in DNA occur at strings of G · C base pairs. Sequence comparisons with the *tk* gene (29) also show occurrences of nucleotide number variability at regions rich in G · C base pairs. Such studies suggest that the HSV DNA polymerase is more error prone in regions of the DNA duplex that are very G+C rich than in other areas.

From intertypic studies between HSV-1 and HSV-2 around 0.60 m.u., little extensive homology was found in the DNA sequences containing the untranslated and untranscribed region between the translational terminator for the 38,000-molecular-weight protein and the terminator for a highly homologous translational reading frame to the right and on the other DNA strand (K. Draper, R. Frink, G. B. Devi, M. Swain, D. Galloway, and E. Wagner, submitted for publication). This suggests that such regions of the HSV genome are under no particular base sequence conservation restraints. There are six regions of changed bases in the 190-nucleotide untranslated region between the termination of the gC reading frame and the initiation for the 17,800-molecular-weight protein translational frame. Two of these involve changes in the number of nucleotides present. This very high frequency of changes is strong evidence for fewer sequence variation constraints in this region than in neighboring regions. This region would appear to contain the promoter for the 730-base mRNA, based on precise mapping data and on the fact that there is a readily detectable TATA box ca. 30 bases upstream of the cap site. Comparative sequence analysis, early and late, of HSV-1 promoters demonstrates that there is little obvious direct sequence conservation (27; Wagner, in press). Therefore, there is no a priori reason to expect strict conservation of sequence in this region. Comparative sequence studies between HSV-1 and HSV-2 (Draper et al., submitted for publication) indicate that there is considerable divergence in the nucleotide sequence in this region between these types. Such a region may prove useful in laboratory analysis of HSV sequence divergence.

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